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Mouse Mammary Tumor Gene *int-3*: a Member of the *notch* Gene Family Transforms Mammary Epithelial Cells

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Expression of a 2.3-kb RNA species is induced in mammary tumors as a consequence of insertional mutagenesis at the *int-3* locus by the mouse mammary tumor virus. The nucleotide sequence and biological activity of this mammary tumor-specific *int-3* RNA species were determined. It contains an open reading frame which encodes a 57-kDa protein. The translated protein possesses six nearly contiguous 32-amino-acid repeats which are related to a similar motif in the *Saccharomyces cerevisiae cdc-10*-encoded cell cycle protein. In addition, the *int-3 cdc-10* repeats are bounded by the PEST amino acid sequence motif which is commonly found in proteins having a rapid turnover and may represent sites for phosphorylation. The *int-3 cdc-10* repeat sequences are 50% identical to a portion of the intracellular domain of the neurogenic *Drosophila notch* gene product. Activation of expression of a recombinant *int-3* genomic DNA fragment encoding the 2.3-kb RNA species in HC11 mouse mammary epithelial cells in vitro induces anchorage-independent growth in soft agar.

Mouse mammary tumorigenesis induced by the mouse mammary tumor virus (MMTV) appears to reflect the clonal outgrowth of mammary epithelial cells containing somatic-mutations induced by viral insertional mutagenesis (37). Constitutive expression of four cellular genes (*int-1*, *int-2*, *hst/K-FGF*, and *int-4*) has been shown to occur in mammary tumors following the integration of a viral genome into adjacent cellular sequences (14, 28, 30, 32). The *int-1* gene (now called *wnt-1*) is the mouse homolog of the *Drosophila* neurogenic gene *wingless* (31). The *int-4* gene (*wnt-3*) is also a member of the same gene family (32). The *int-2* and *hst/K-FGF* genes are members of the fibroblast growth factor (*FGF*) gene family (11, 13, 41). Normally these genes are expressed only during embryonic development, except in the case of *wnt-1*, which is also expressed in the adult testis (22). The mechanism by which the virus activates gene expression appears to reflect primarily the effect of *cis*-acting enhancer sequences within the MMTV long terminal repeat (LTR) element on the transcription-regulatory sequences of the target cellular gene. We have previously described a common insertion site for the MMTV in mouse mammary tumors, designated *int-3* (19). One consequence of viral integration at *int-3* is activation of expression of a 2.3-kb RNA species corresponding to adjacent cellular sequences 3′ of the viral insertion sites. This unique RNA species has not been detected in tumors in which the *int-3* locus is unaffected or in normal adult tissues. We now report that the nucleotide sequence of this *int-3* RNA species is similar to the intracellular domain of the neurogenic *notch* gene (9, 16, 38, 39) and demonstrate that its expression alters the growth properties of the HC11 mouse mammary epithelial cell line (4) in culture.

The organization of the *int-3* locus is shown in Fig. 1. It is rearranged by MMTV integration in approximately 20% of virus-induced Czech II mouse strain mammary tumors (19). In all of the tumors having an MMTV-induced rearrangement of the *int-3* locus, the transcriptional orientation of the integrated viral genomes are all in the same direction and the integration site is restricted to a 500-bp region of the locus (Fig. 1). To characterize the properties of the activated *int-3* gene product and the mechanism of activation by MMTV, a flanking genomic DNA fragment (Fig. 1, probe A) was used to probe an *int-3*-positive mammary tumor cDNA library. Six cDNA clones were obtained, and one of these (clone 241) was 2.3 kb long. The nucleotide sequence of clone 241 was determined by dideoxy-chain termination with Sequenase (U.S. Biochemicals) by using the manufacture-suggested protocol (Fig. 2). Analysis of the clone 241 nucleotide sequence indicates that transcription of the *int-3* cellular sequences in this tumor was initiated from within the 3′ MMTV (Czech II) LTR. The 5′ 96 bp of the cDNA corresponds to the U5 region of the viral LTR sequence, while the remainder of the cDNA represents eight exons located within 4.8 kbp of cellular DNA sequences 3′ of the viral insertion site (Fig. 1 and 2). This finding demonstrates that while the primary mechanism of activation of the other *int* genes by MMTV is enhancer insertion, *int-3* expression is activated by promoter insertion.

The nucleotide sequence of the cellular flanking sequences in the cDNA was compared to those in the GenBank data base. The *int-3* sequence was most similar to the region of the *notch* gene from *Drosophila melanogaster*, *Xenopus laevis*, rats, and humans (57 to 63% identity), which encodes the intracellular domain of the protein (9, 16, 38, 39). The *int-3* clone 241 cDNA contains an open reading frame which encodes a protein of 552 amino acids. This protein is initiated by an ATG codon that is 26 bp 3′ of the host-virus junction and is preceded by a sequence similar to the Kozak box (24) which frequently precedes a translation initiation codon. BEST FIT analysis (12) demonstrated that one distinctive feature of the *notch*-encoded protein which has been conserved in the *int-3*-encoded protein (64 to 67% similarity, 50 to 52% identity) is six nearly contiguous copies of a 33-amino-acid repeat sequence (Fig. 3A and B). This repeat motif was first recognized in the *cdc-10* (3)- and *SWI6* (7)-encoded cell cycle-regulatory proteins (38 and 36% identical, respectively) of yeasts (Fig. 3B) and has also been found in several other proteins which have roles in cell determination and cell cycle control, including the β subunit of heteromeric DNA-binding protein GABP (25), the *NFκB/KBF-1* transcription factor (5, 23) and regulatory subunit

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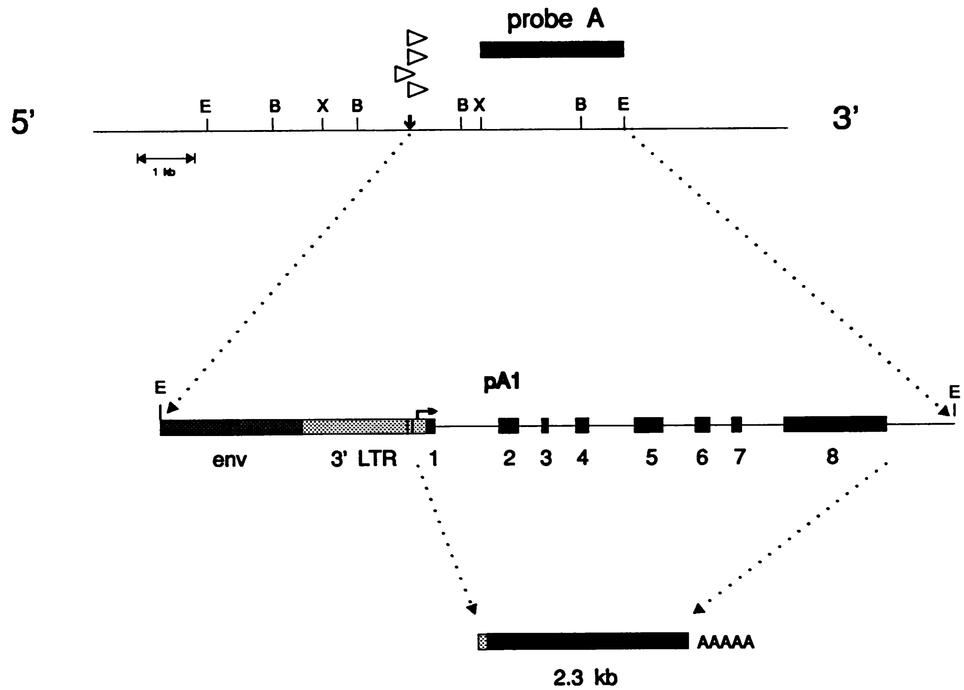


FIG. 1. Diagram of the *int-3* locus and tumor transcript. The top line represents the uninterrupted locus, indicating points of viral insertion and appropriate restriction sites. The open triangles reflect the transcriptional orientation and insertion site of the integrated MMTV genomes in five independent tumors (see text). Probe A (described in the text) is a genomic clone from *Xba*I to *Eco*RI (E, *Eco*RI; B, *Bam*HI; X, *Xba*I). The middle line represents 7.5-kb of *Eco*RI-to-*Eco*RI cloned DNA from an *int-3*-positive tumor (19). This clone contains partial MMTV sequences from the 3' side of the integrated virus along with the flanking cellular sequences that encode the *int-3* tumor transcript. The black boxes represents the eight exons inferred from the cDNA and genomic DNA sequences. The black arrow indicates the start of transcription. The bottom line shows the 2.3-kb tumor RNA transcript originally described in reference 19. The previously published map (19) of the *int-3* locus incorrectly positioned probe C, suggesting that the 2.3-kb *int-3* RNA corresponds exclusively to cellular sequences 5' of the viral integration site. Nucleotide sequence analysis has shown that probe C is actually a *Bam*HI-*Xba*I fragment located 3' of the viral insertion site. The stippled box at the 5' end of the activated *int-3* RNA represents the portion of the U5 region of the LTR that is also transcribed.

MAD-3 (21), the *BCL-3* proto-oncogene product (29), the protein encoded by sex-determining gene *fem-1* of *Caenorhabditis elegans* (35), the human erythrocyte ankyrin protein (26), and the *lin-12* and *glp-1* gene products of *C. elegans* (40), both of which are transmembrane proteins that are involved in cellular determination during development. The *cdc-10/SWI6* repeats in these other proteins were much less similar (28 to 33% identity) to the *int-3* repeat sequences (Fig. 3B).

Another feature common to the activated *int-3*- and *notch*-encoded proteins is the presence of two PEST sequences (33). PEST sequences are defined by clusters of proline (P), glutamic acid (E), serine (S), and threonine (T) residues and are commonly found in cell-regulatory proteins which are rapidly degraded or may represent potential protein phosphorylation sites. As in the *notch*-encoded protein (39), the PEST sequences in the *int-3*-encoded protein lie on either side (residues 100 to 129 and 511 to 527) of the *cdc-10/SWI6* repeat sequences (Fig. 2). A similar arrangement of PEST sequences and *cdc-10/SWI6* repeat sequences is also present in the intracellular domain of the *lin-12*-encoded cell fate-determining protein (40). We were curious as to whether the position of PEST sequences relative to *cdc-10/SWI6* repeats is a general structural feature of proteins containing these repeat sequences. Although all of the other proteins containing *cdc-10/SWI6* repeats, except *fem-1*, also contain PEST sequences, only in the *BCL-3*- and *NFκB/KBF-1*-encoded proteins are the *cdc-10/SWI6* repeats similarly bounded by PEST sequences. This conserved association suggests an essential role for the PEST motif in these *cdc-10/SWI6*-containing proteins.

The similarity between the *int-3* and *notch* gene nucleotide sequences, as well as the temporal organization of amino acid sequence motifs between the activated *int-3*-encoded protein and the intracellular portion of the *notch* gene product, suggests that *int-3* is a member of the *notch* gene family. The extracellular domain of the *notch*-encoded protein contains multiple cysteine-rich epidermal growth factor-like repeats (39). Although the activated *int-3*-encoded protein does not appear to have an extracellular domain, nucleotide sequence analysis of genomic cellular DNA adjacent to the 5' end of MMTV genomes integrated in the *int-3* locus revealed three potential exons which encode five epidermal growth factor-like repeats and three *lin-12–notch* repeats (40; unpublished data). However, two characteristics of the *int-3* gene suggest that it is not the murine homolog of *notch*. (i) The activated *int-3* gene shares only 63% identity with the corresponding regions of the rat (38) and human *notch* (16) genes, whereas the rat and human *notch* genes are 84% identical in this region. (ii) The *int-3*-encoded protein lacks the polyglutamine sequences referred to as the opa or M repeat that is characteristically found near the C terminus of the *notch*-encoded protein (39). We conclude, therefore, that *int-3* represents a new member of the *notch* gene family.

The means by which *notch* affects cell fate decisions is

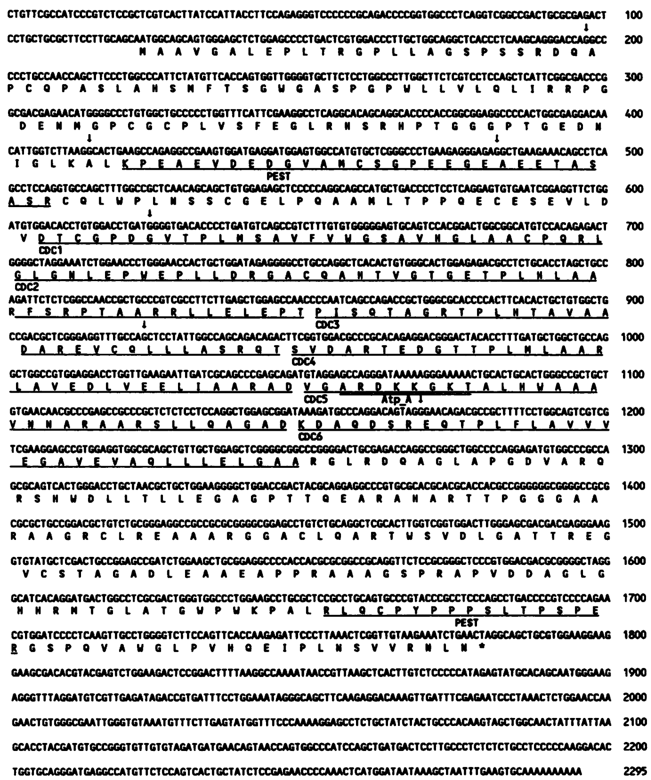


FIG. 2. Complete nucleotide sequence of the 2.3-kb *int-3* tumor transcript. The first 96 bases are derived from the 3′ viral LTR. Intron breaks are represented by small arrows above the start of the next exon. The deduced amino acid sequence appears below the nucleotide sequence. The two flanking PEST sequences and the *cdc-10* repeat motifs are underlined and identified. A potential ATP/GTP binding site motif is doubly underlined. Abbreviations for amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

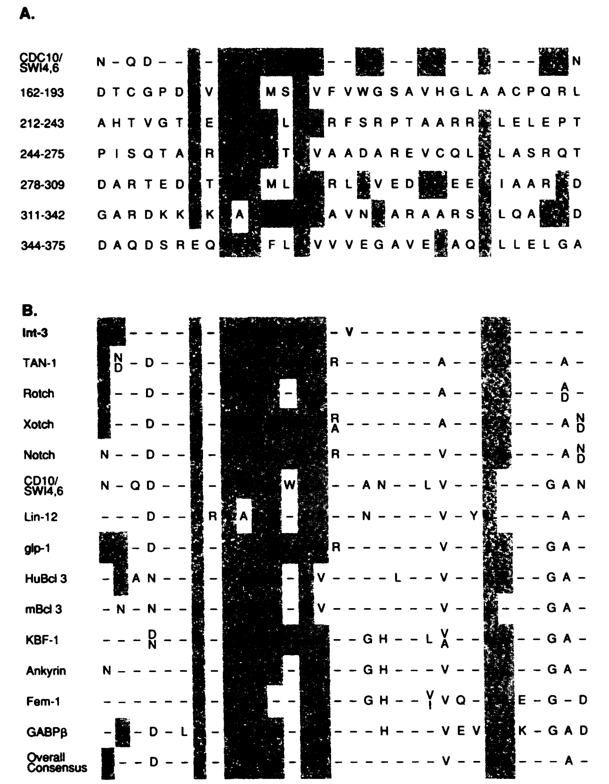
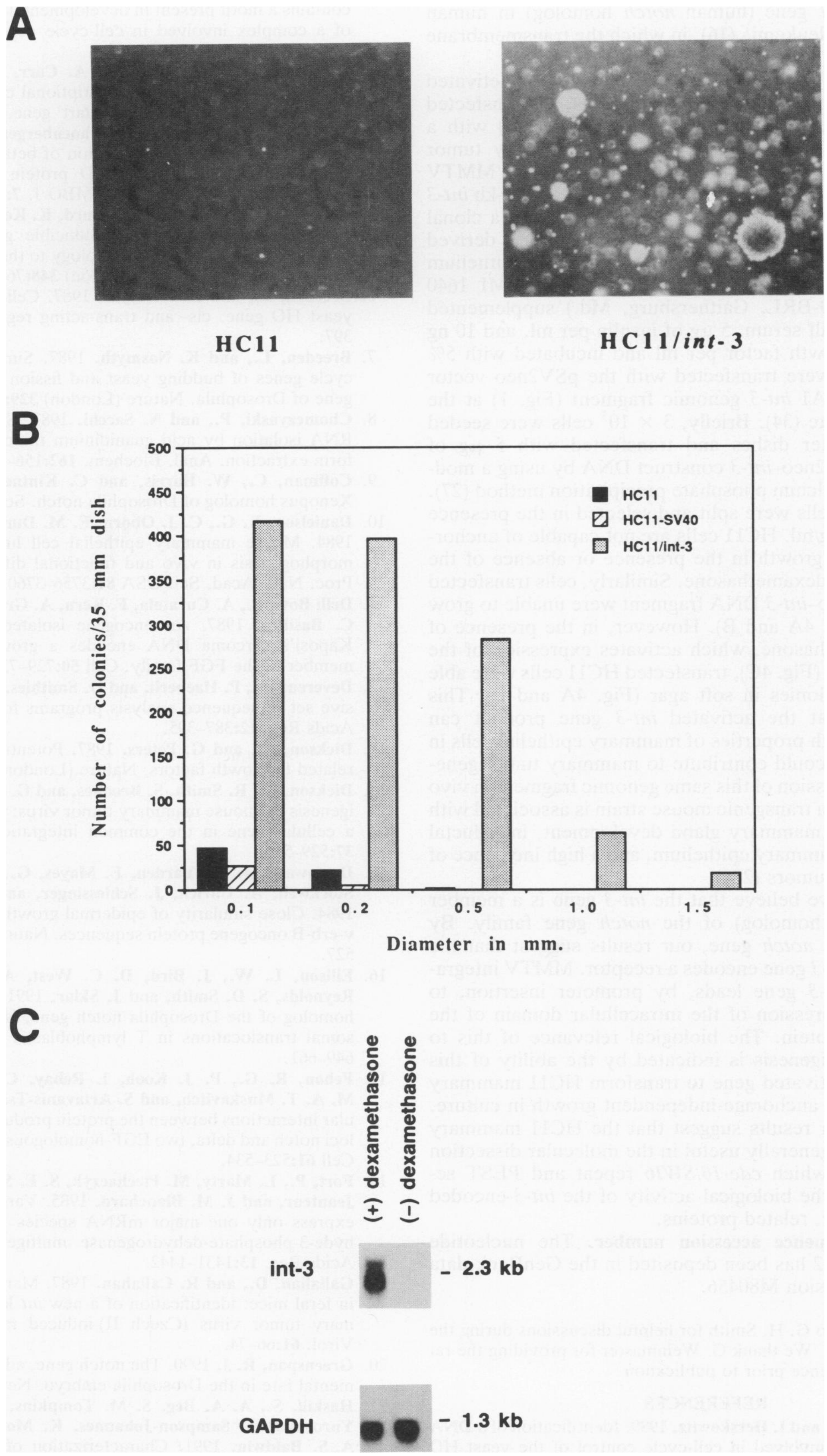


FIG. 3. *cdc-10/SWI6* repeat sequence in *int-3* and consensus alignment with other *cdc-10*-bearing proteins. (A) The consensus sequence for *cdc-10/SWI4/SWI6* was compared to the *int-3* amino acid sequences for similarity by using the Compare program (12). The consensus sequence for *int-3* was determined by aligning all of the repeats and determining where a plurality for a specific amino acid exists. The amino acid residues that share homology with the consensus sequence are highlighted. (B) The 32-amino-acid *cdc-10/SWI6* consensus sequence from *int-3* compared with related consensus repeat sequences from other proteins. Repeat consensus sequences for the other proteins were either published or generated by using the same criteria as for *int-3*. The highlighted amino acids are those that are in common with the *int-3* consensus sequence. The amino acid abbreviations are the same as those in Fig. 2.

unclear. Absence of the *notch* gene product leads to expansion of one cell type at the expense of another (20). The potential importance of the extracellular domain of the *notch*-encoded protein in mediating specific adhesive interactions between cells during development has represented a major focus of attention (17, 20). However, it has also been suggested that the *notch*-encoded protein is a receptor and that its intracellular domain plays a role in signal transduction in response to ligand binding to the extracellular domain (17). The region of the intracellular domain which represents a primary candidate for participation in the protein-protein interactions that are commonly involved in signal transduction is the *cdc-10/SWI6* repeat. In this regard, the *cdc-10/SWI6* repeat sequences in several proteins have recently been implicated in cytoplasmic protein-protein (17, 26, 40) and nuclear protein-protein and protein-DNA (1, 2, 5, 6, 21, 23, 36) interactions.

If the uninterrupted *int-3* gene encodes a transmembrane protein (receptor), then a primary consequence of MMTV integration in this locus is activation of expression of its intracellular domain and its release from extracellular control. This is consistent with the apparently high level of selection for the transcriptional orientation of the integrated viral genome and also the site of integration in the *int-3* locus (Fig. 1). In this scenario, MMTV-induced activation of *int-3* has the same molecular consequences as in the v-*erbB*

FIG. 4. Growth and expression in HC11 cells transfected with the *int-3* gene. (A) Anchorage-independent growth by HC11 and HC11/*int-3* cells was assayed in soft agar containing dexamethasone (10^{-7} M) (27). Representative morphology was photomicrographed after 10 days of growth. Colonies are shown at a magnification of ×37.5. (B) Numbers and sizes of colonies were measured by using an Artek 800 colony counter after 14 days of growth in soft agar containing dexamethasone at 10^{-7} M. SV40, simian virus 40. (C) Induced expression of the *int-3* tumor transcript in transfected HC11 cells grown from colonies. Total RNA was extracted from 10^7 cells by the guanidine thiocyanate method (8). A 10-μg sample of RNA was separated on a 1% agarose-formaldehyde gel and transferred to a Genetran[45] nylon membrane (Plasco, Woburn, Mass.). The blot was hybridized with a randomly primed ^{32}P-labeled *int-3* cDNA probe. After washing and exposure of the blot to X-ray film, it was stripped and reprobed with the control gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 18).


A
HC11
HC11/int-3
B
Number of colonies/35 mm dish
HC11
HC11-SV40
HC11/int-3
Diameter in mm.
0.1
0.2
0.5
1.0
1.5
500
450
400
350
300
250
200
150
100
50
0
C
(+) dexamethasone
(−) dexamethasone
int-3
2.3 kb
GAPDH
1.3 kb

oncogene (15) or the t(7;9)(q34;q34.3) translocations that affect the *TAN-1* gene (human *notch* homolog) in human T-lymphoblastic leukemia (16), in which the transmembrane and intracellular domains are overexpressed.

To determine the biological consequences of activated *int-3* expression on mammary epithelial cells, we transfected the HC11 mouse mammary epithelial cell line (4) with a genomic DNA fragment from a mouse mammary tumor which contains a portion of the MMTV *env* gene, the MMTV LTR, and the cellular sequences from which the 2.3-kb *int-3* RNA is transcribed (Fig. 1). The HC11 cell line is a clonal derivative of the COMMA D cell line, which was derived from normal midpregnancy BALB/c mammary epithelium (10). The HC11 cell line was propagated in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, Md.) supplemented with 10% fetal calf serum, 5 μg of insulin per ml, and 10 ng of epidermal growth factor per ml and incubated with 5% CO_2. The cells were transfected with the pSV2neo vector containing the pA1 *int-3* genomic fragment (Fig. 1) at the *Eco*RI cloning site (34). Briefly, 3×10^5 cells were seeded in 60-mm-diameter dishes and transfected with 5 μg of pSV2neo or pSV2neo–*int-3* construct DNA by using a modification of the calcium phosphate precipitation method (27). After 24 h, the cells were split and selected in the presence of G418 at 400 μg/ml. HC11 cells are not capable of anchorage-independent growth in the presence or absence of the steroid hormone dexamethasone. Similarly, cells transfected with the pSV2neo–*int-3* DNA fragment were unable to grow in soft agar (Fig. 4A and B). However, in the presence of 10^{-7} M dexamethasone, which activates expression of the 2.3-kb *int-3* RNA (Fig. 4C), transfected HC11 cells were able to form large colonies in soft agar (Fig. 4A and B). This demonstrates that the activated *int-3* gene product can perturb the growth properties of mammary epithelial cells in a manner which could contribute to mammary tumorigenesis. In fact, expression of this same genomic fragment in vivo as a transgene in a transgenic mouse strain is associated with arrest of normal mammary gland development, intraductal hyperplasia of mammary epithelium, and a high incidence of focal mammary tumors (22a).

In summary, we believe that the *int-3* gene is a member (not the mouse homolog) of the *notch* gene family. By analogy with the *notch* gene, our results suggest that the uninterrupted *int-3* gene encodes a receptor. MMTV integration into the *int-3* gene leads, by promoter insertion, to activation of expression of the intracellular domain of the *int-3*-encoded protein. The biological relevance of this to mammary tumorigenesis is indicated by the ability of this portion of the activated gene to transform HC11 mammary epithelial cells to anchorage-independent growth in culture. Furthermore, our results suggest that the HC11 mammary cell line may be generally useful in the molecular dissection of the function which *cdc-10/SWI6* repeat and PEST sequences play in the biological activity of the *int-3*-encoded protein and other, related proteins.

Nucleotide sequence accession number. The nucleotide sequence in Fig. 2 has been deposited in the GenBank data base under accession M80456.

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